Young Infants Can Develop Protective Levels of Neutralizing Antibody after Infection with Respiratory Syncytial Virus

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Humoral immunity protects against severe respiratory syncytial virus (RSV) disease, but the range and magnitude of antibody responses in RSV-naive children after RSV infection have not been completely defined. We evaluated RSV-neutralizing antibody and immunoglobulin G responses to RSV F and G glycoproteins in 65 RSV-naive Navajo and White Mountain Apache children aged 0–24 months who were hospitalized with RSV infection. In these children, antibody responses developed against RSV F and G and the central conserved region of RSV G. Twenty-seven of 41 infants <6 months old developed reciprocal $\log_2$ RSV neutralizing antibody titers ⩾8.0, which correlate with protection of the lower respiratory tract. Multivariate analysis demonstrated that the level of preexisting neutralizing antibody at infection, not age, was the most important factor influencing this response. RSV can induce substantial neutralizing antibody responses in young infants when the titer of preexisting antibodies is low.

Respiratory syncytial virus (RSV) is the most important cause of viral lower respiratory tract illness in infants and children worldwide [1, 2]. Premature infants and infants with congenital heart or lung disease are at increased risk for severe RSV disease [3–6], and passive prophylaxis with administration of RSV antibody is recommended for these groups [7, 8]. However, the majority of infants hospitalized each year with RSV lower respiratory tract illness do not have these risk factors. For this reason, development of an RSV vaccine for infants remains a priority.

Protection against severe RSV disease can be provided by virus-specific neutralizing antibody, as has been demonstrated by passive-transfer experiments in rodents [9, 10] and by the clinical experience with prophylaxis involving RSV intravenous immune globulin [11] and palivizumab [12] in high-risk infants. However, the range and magnitude of antibody responses to RSV in RSV-naive infants and children experiencing naturally acquired wild-type (wt) RSV infection have not been fully explored. Specifically, it is generally believed that very young infants may not be capable of producing high titers of neutralizing antibody in response to RSV infection or immunization [13]. In addition, the antigenic specificity of the antibody response to RSV merits further investigation. Young infants who received *cpts*248/404, a live attenuated RSV vaccine candidate, developed antibody responses directed primarily against the RSV G glycoprotein [14]. Because this surface glycoprotein is composed of conserved and highly variable regions [2, 15, 16], it would be useful for future vaccine development to determine whether antibody responses are directed primarily toward conserved regions of RSV G.

Our previous epidemiologic study of RSV in Navajo and White Mountain Apache (WMA) American Indian

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infants [17] enabled us to explore the specificity and magnitude of antibody responses in infants and children naturally infected with RSV and to determine the effects of age and preexisting (likely maternally derived) RSV neutralizing antibody on these responses.

METHODS

Study population: American Indian children naturally infected with wt RSV. Serum samples from 82 WMA or Navajo children living on reservations in Arizona or New Mexico who were hospitalized with RSV infections from 1997 to 2000 were analyzed for this study. These 0–24-month-old children had been enrolled in a surveillance study to determine rates of hospitalization for RSV disease [17]. Written informed consent was obtained from a parent before each child's enrollment in this study. This study was approved by the Johns Hopkins institutional review board (IRB), the Phoenix area IRB, the Navajo Nation IRB, and the WMA Tribal Council. The diagnosis of RSV infection was made by rapid antigen test (TestPack EIA [Abbott] or Directigen [Becton Dickinson]). Serum samples were obtained at the time of hospitalization and 4–13 weeks later (mean, 6.6 weeks later). Seventeen children ⩾6 months of age with neutralizing antibody titers >1:40 in their acute-stage serum samples were classified as RSV seropositive, and those serum samples were excluded from further analysis; thus, serum samples from 65 RSV-naive infants and children were available for testing.

For purposes of comparison, serum samples from 46 Navajo or Apache infants not infected with RSV were also assayed for RSV neutralizing antibodies. These children were born after the RSV season and had serial serum samples obtained during their participation in a pneumococcal conjugate vaccine efficacy trial from 1997 to 2000 [18].

Immunologic assays. Serum samples were tested for neutralizing antibody to RSV A2 using a 60% complement-enhanced plaque reduction neutralization assay [19]. IgG antibodies to RSV F and G glycoproteins were measured by ELISA as described elsewhere [14], using purified F and G glycoproteins provided by V. Randolph (Wyeth Vaccines). IgG antibodies to RSV G peptides and to the cysteine noose region of RSV G were also measured by ELISA. Peptides spanning the central region of the ectodomains of the RSV G_A and G_B proteins were previously synthesized with biotin-serine-glycine-serine-glycine at their amino termini and were provided by R. M. Hendry (Wyeth Vaccines Research). We limited our analysis to this central region, because the particular strains that infected the children were unknown, and the central region is the most conserved. The peptides were generated using consensus sequences for multiple RSV A and RSV B subgroup strains, respectively [20]. Each peptide contained 15 aa with a 5-aa overlap and offset, as described elsewhere [20]; they were used as a pool spanning ^{144}S to T^{198} for RSV A and ^{148}T to P^{202} for RSV B. Also used as antigens for ELISA were 26-aa biotinylated peptides representing the cysteine noose regions of RSV G_A and G_B (aa 164–189) [20].

Table 1. Respiratory syncytial virus (RSV) antibody assays in children <6 months and 6–24 months old.

		IgG antibody to RSV						
							Cysteine noose	
Age	Neutralizing antibody	F	G_A	G_B	$G3_A$	$G3_B$	G_A	G_B
<6 months	41	37	36	35	25	26	26	26
6–24 months	24	21	21	21	18	18	17	18

NOTE. Data are the no. of serum sample pairs tested by the indicated assay.

For the G peptide IgG ELISA, Immulon 4 plates (Dynatech Laboratories) were coated with 1 μg/well NeutraVidin (Molecular Probes) and pools of biotinylated peptides (0.1 μg/well in PBS). Serum samples were added in duplicate to antigen-coated and uncoated wells at starting dilutions of 1:20. Bound antibody was detected using goat anti–human IgG peroxidase conjugate (Kirkegaard and Perry) and 3,3',5,5'-tetramethylbenzidine (Bio-Rad Laboratories) as substrate. Optical densities (ODs) were read at 450 nm, and the OD for the corresponding blank well without antigen was subtracted to obtain the final value. To minimize interassay variability, a reference serum was used in each run, and ODs were multiplied by a correction factor (mean titer of reference serum/reference serum titer on the individual plate). The end-point dilution at 0.2 OD was calculated using log/log transformation of the corrected OD versus the sample dilution. The titer is the reciprocal of the end-point dilution.

Prioritization of assays. Because limited volumes of serum samples were available from these young children, a stepwise procedure was used to prioritize the antibody assays. Neutralizing antibody assays were performed on serum samples from all subjects. Samples were tested for IgG antibody to RSV F, G, and G peptides according to the age of the subject and volume of serum available for testing.

For children ⩾6 months of age, serum samples were tested for IgG antibody to the RSV F, G_A, and G_B glycoproteins; the G_A3 and G_B3 peptides; and the G_A and G_B cysteine noose peptides. ELISAs to detect IgG antibody to group 3 G_A and G_B peptides and the G_A and G_B cysteine noose regions were performed for all infants <6 months old from whom sufficient serum was available. When the volume of serum was insufficient to perform all peptide assays, G_A peptide assays were performed on serum samples with higher titers of antibody to the G_A glycoprotein, and G_B peptide assays were performed on serum samples with higher titers of antibody to the G_B glycoprotein. The number of serum sample pairs tested in each assay is indicated by age group in table 1.

Statistical analysis. All antibody titers are expressed as reciprocal $\log_2$ values. Fisher's exact test was used to compare proportions, the Mann-Whitney *U* test was used to compare titers between groups, and correlation coefficients with significance levels were calculated using Spearman rank-order correlation. Simple and multivariate linear regression models were used to examine the association between change in neutralizing antibody titer and hypothesized indicators. Analyses were performed using SAS (version 8.2; SAS Institute) and Stata (version 7.0; StataCorp) software.

RESULTS

Response to RSV F and G glycoproteins and to RSV G peptides. As shown in table 2, IgG responses to both RSV F and G were observed in most infants and children naturally infected with RSV, and the frequency and magnitude of responses to F and G were quite similar. These infants and children developed significantly higher titers of antibody to G_B than to G_A (for children <6 months old, 13.5 vs. 11.8 [$P = .006$]; for children 6–24 months old, 14.1 vs. 12.2 [$P = .04$]) (table 2). Possible explanations for this observation include more frequent RSV subgroup B infections in this population during the period of observation or intrinsic properties of the purified glycoproteins causing higher titers of antibody to G_B. Infants and children naturally infected with wt RSV had frequent responses to $G3_A$ or $G3_B$ peptides and the G_A or G_B cysteine noose (table 3), and the magnitude of the postinfection antibody titer to the G_A cysteine noose correlated with the postinfection antibody titer to the intact G_A glycoprotein ($r = .54$; $P < .001$).

RSV neutralizing antibody responses. As shown in table 2, neutralizing antibody responses were frequently observed in naturally infected children ≥6 months of age and in infants <6 months of age. Surprisingly, the magnitude of the convalescent-stage neutralizing antibody titers was significantly greater in these very young infants than in the older children (8.6 vs. 7.1; $P = .017$).

The relationship between acute- and convalescent-stage neutralizing antibody titers in naturally infected infants is shown in figure 1. Even in infants 0–90 days of age, infection with wt RSV generally induced high titers of neutralizing antibody in those with low levels of preexisting antibody; among those with acute-stage titers <5.3, the mean postinfection titer was 8.2 (figure 1*A*). Similar responses were observed in infants 91–180 days of age (figure 1*B*).

Previous studies have shown that young infants often fail to develop neutralizing antibody responses after RSV infection [21–23], a finding variously attributed to immunologic immaturity and/or suppression of the infant antibody response by maternally derived antibody [2, 24, 25]. For this reason, the relationships between the change in neutralizing antibody titer, the level of neutralizing antibody in the acute-stage samples, and age at the time of infection were explored by linear regression analysis. In the univariate analyses, there was a significant negative association between the level of neutralizing antibody in the acute-stage samples and the change in neutralizing antibody titer between the acute- and convalescent-stage samples ($b = -.43$; $P = .02$) but no significant association between age and change in titer. A multivariate analysis was performed, including level of antibody in the acute-stage samples, age, and time interval between acute- and convalescent-stage samples; again, only the level of antibody in the acute-stage samples was significant in this analysis ($b = -.42$ [95% confidence interval, $-.79$ to $-.05$]; $P = .03$) after controlling for age and time interval, and no interaction was observed between age and level of antibody in the acute-stage samples. These data suggest that the level of preexisting maternally derived antibody in young infants, rather than age, is the most important factor influencing the neutralizing antibody response to RSV infection.

Compared with other US populations, American Indian and Alaska Native infants are at increased risk for RSV-related hospitalization [17, 26–28]. In this study, hospitalized infants <6 months old had relatively low levels of neutralizing antibody in their acute-stage serum samples (mean titer, 5.7) (figure 1), which is a known risk factor for severe RSV disease in young infants [29–31]. For this reason, we wanted to determine whether young WMA or Navajo infants hospitalized with RSV were particularly likely to be RSV seronegative (titer of <5.3) or whether low levels of neutralizing antibody were commonly found this population of young infants. As described in Methods, we also measured titers of RSV neutralizing antibodies in Navajo and WMA infants born outside the RSV season, who were used as a comparison group. As shown in figure 2, 16 of 24 infants ≤120 days of age who were hospitalized for RSV disease had neutralizing antibody titers <5.3 (figure 2*A*), compared with only 4 of 53 infants ≤120 days of age in the comparison group (figure 2*B*) ($P < .001$). For infants >120 days of age, no significant differences were observed in the proportions of infants with low titers of RSV neutralizing antibody, most likely because of the rate of decay of maternally derived antibody. These data suggest that, as in other populations, the subset of young American Indian infants with very low levels of maternally derived RSV neutralizing antibody may be at increased risk for severe RSV disease.

DISCUSSION

RSV remains an important cause of morbidity in infants. Although a vaccine against RSV is not yet available, significant progress has been made in recent years, and 2 live attenuated candidate vaccines have been evaluated in the target population (i.e., infants 1–2 months of age) [14, 32]. As additional candidate vaccines are developed and tested in this age group, it will be important to understand the capacity of young infants to re-

Table 2. Serum IgG antibody responses to respiratory syncytial virus (RSV) F and G glycoproteins and RSV neutralizing antibody responses in previously RSV-naive infants and children.

	F			G_A			G_B			G_A or G_B,	RSV neutralizing antibody titer		
Age	Pre	Post	⩾4-fold rise	Pre	Post	⩾4-fold rise	Pre	Post	⩾4-fold rise	⩾4-fold rise	Pre	Post	⩾4-fold rise
<6 months	9.6 ± 3.9	14.2 ± 3.8	26/37	10.6 ± 3.3	11.8 ± 3.0	14/36	11.0 ± 3.2	13.5 ± 2.8	17/35	26/36	5.7 ± 2.3	8.6 ± 2.8	25/41
6–24 months	7.4 ± 2.4	15.4 ± 2.9	19/21	7.7 ± 2.8	12.2 ± 3.0	17/21	8.5 ± 2.2	14.1 ± 2.8	16/21	19/20	3.7 ± 1.0	7.1 ± 2.4	16/24

NOTE. All titers are expressed as mean ± SE reciprocal $\log_2$ values. Titers were measured by ELISA. The proportions of subjects with a ⩾4-fold rise between pre- and postinfection titers are given as the no. of serum sample pairs with a ⩾4-fold rise per the no. of sample pairs tested by the indicated assay (see Methods for an explanation of the prioritization of assays).

Table 3. Serum IgG antibody responses to respiratory syncytial virus (RSV) G peptides in previously RSV-naive infants and children.

	$G3_A$			$G3_B$			$G3_A$ or $G3_B$, ⩾4-fold rise	G_A cysteine noose			G_B cysteine noose			G_A or G_B cysteine noose, ⩾4-fold rise
Age	Pre	Post	⩾4-fold rise	Pre	Post	⩾4-fold rise		Pre	Post	⩾4-fold rise	Pre	Post	⩾4-fold rise	
<6 months	8.0 ± 2.3	8.9 ± 2.8	9/25	7.5 ± 1.6	10.4 ± 2.3	18/26	21/33	9.5 ± 3.1	11.3 ± 3.8	11/26	8.6 ± 2.3	12.6 ± 3.4	16/26	23/34
6–24 months	6.5 ± 1.4	8.5 ± 2.0	8/18	7.1 ± 2.0	11.8 ± 3.2	13/18	15/20	5.7 ± 1.3	10.4 ± 4.3	11/17	8.3 ± 2.9	12.8 ± 3.1	13/18	16/20

NOTE. All titers are expressed as mean ± SE reciprocal $\log_2$ values. Titers were measured by ELISA. The proportions of subjects with a ⩾4-fold rise between pre- and postinfection titers are given as the no. of serum sample pairs with a ⩾4-fold rise per the no. of sample pairs tested by the indicated assay (see Methods for an explanation of the prioritization of assays).

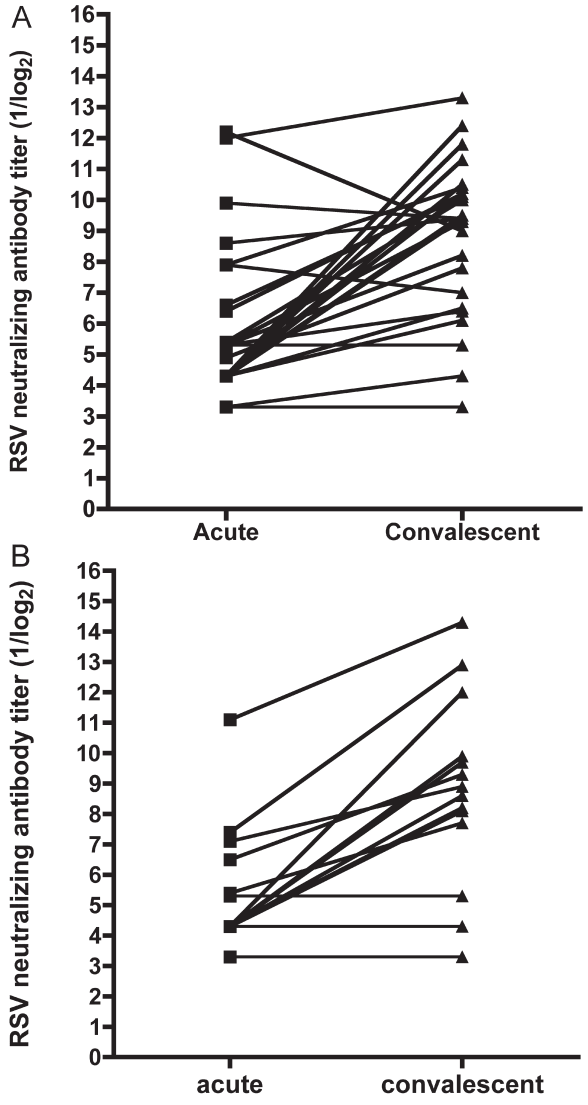

Figure 1. Relationship between acute- and convalescent-stage titers of respiratory syncytial virus (RSV) neutralizing serum antibody in hospitalized Navajo and White Mountain Apache infants 0–90 *(A)* or 91–180 *(B)* days old. Acute-stage specimens were obtained during hospitalization, and convalescent-stage specimens were obtained 4–13 weeks later (mean, 6.6 weeks later).

spond to natural infection with RSV or immunization with live attenuated vaccines. Because serum antibody is an important mediator of protection against severe RSV disease [2, 11, 12], this study focused on serum antibody responses in a large group of naturally infected infants and children. This cohort had substantially lower titers of maternally derived antibody than were typically found in other infants in the local population (figure 2) or in infants of comparable age enrolled in our vaccine studies [14, 32], providing an opportunity to evaluate primary antibody responses under conditions in which the confounding factor of maternal antibody–mediated immunosuppression was less prominent.

Although the present study demonstrates strong and equivalent antibody responses to RSV F and G glycoproteins in young infants, our previous studies with the live attenuated RSV vaccine *cpts*248/404 showed that this population preferentially responded to the RSV G glycoprotein [14]. For this reason, we used biotinylated peptides spanning the central conserved region of RSV G to define further the specificity of the response to RSV G. We found that responses to the central conserved region of G (residues 164–189) frequently occurred in naturally infected children, could be detected using either 15-aa linear peptides or a larger 26-aa peptide spanning the entire region, and correlated with responses to the intact RSV G glycoprotein. Earlier studies have described antibody responses to this central conserved region [33, 34], although in smaller numbers of children and not with same frequency described here. Others have described responses to linear epitopes in the variable carboxy-terminal region of RSV G that are highly dependent on the RSV genotype [35]; we were not able to assess these responses because the genotypes of the infecting strains were unknown.

The cysteine noose region of RSV G is highly conserved among RSV subgroup A and B isolates [36] but is not required for viral replication in vitro or in vivo [37]. However, this region appears to have potent immunomodulatory effects: it contains a CX3C chemokine motif that can bind to CX3CR1 and alter cellular trafficking patterns [38] and induces Th2 responses in mice [39, 40], but it is also critical for induction of cytotoxic T lymphocyte responses in mice [41]. We have previously shown that adults and children infected with wt RSV or receiving live attenuated RSV vaccine candidates produce IgG antibodies that inhibit binding of RSV G to CX3CR1 and RSV G–mediated leukocyte chemotaxis [42]. The present study provides complementary information regarding the specificity of the RSV G response; it is likely that these inhibitory effects are mediated through IgG that binds to the central conserved cysteine noose region of RSV G.

In this study, we also examined the neutralizing antibody responses of very young infants who were naturally infected with wt RSV. In contrast to previous studies that demonstrated low levels of RSV neutralizing antibody after RSV infection in young infants [13, 21, 23, 43], we found that many of the very young infants who were hospitalized with RSV infection produced large quantities of RSV neutralizing antibody, often meeting or exceeding the 8.0–9.0 titers typically associated with protection of the lower respiratory tract [11, 43]. In a multivariate analysis, we found that the titer of neutralizing antibody in the acute-stage serum sample (presumably acquired through passive transfer of maternal antibody), not age, was the most important determinant of this response. Our findings may differ from those of previous studies because we were able to evaluate a relatively large cohort of young infants hospitalized with RSV infection and because these hospitalized infants had substantially lower titers of maternally derived antibody than typically found

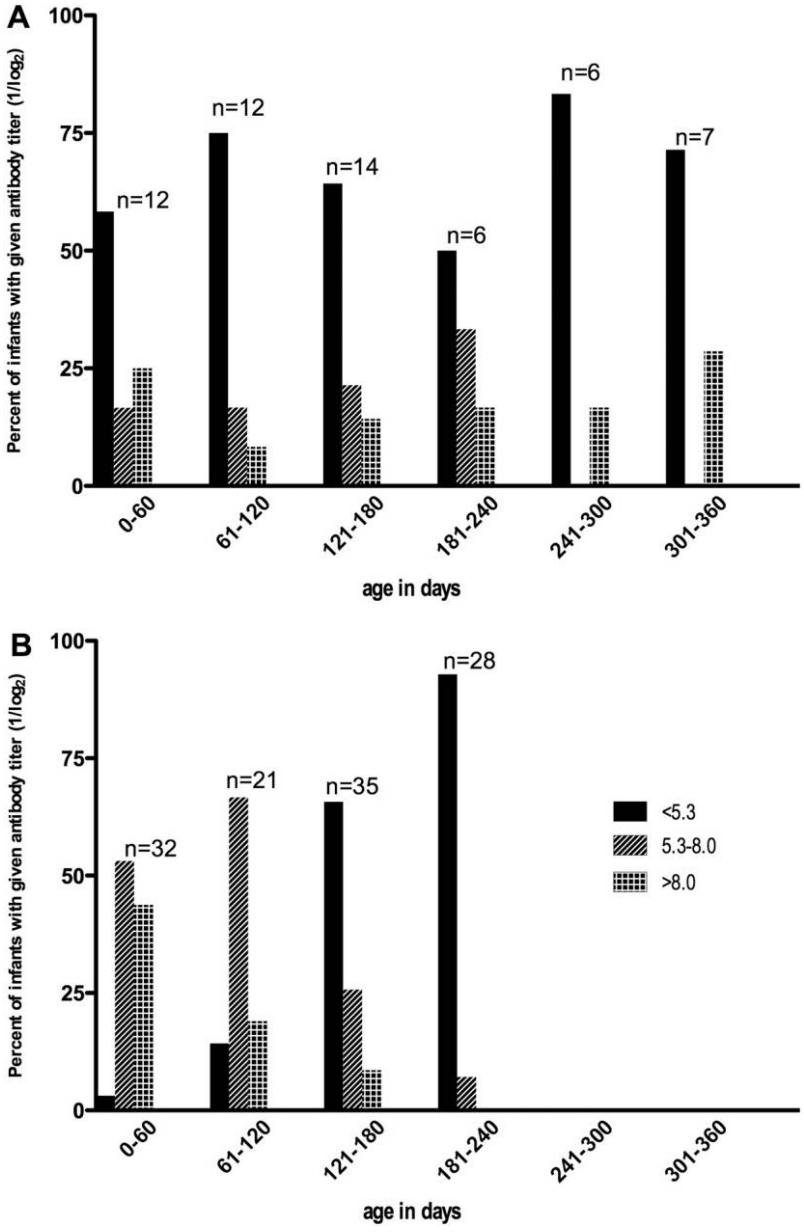

Figure 2. Percentage of Navajo or White Mountain Apache infants with low (<5.3), intermediate (5.3–8.0), or high (>8.0) titers of acute-stage respiratory syncytial virus (RSV) neutralizing serum antibody at the age intervals shown. Panel A summarizes data from infants hospitalized with RSV infection, and panel B summarizes control data from infants born after the RSV season who had serum specimens obtained during a pneumococcal conjugate vaccine efficacy trial.

in other infants in the local population (figure 2) or in non–American Indian infants of comparable age enrolled in vaccine studies [14, 32]. That the infants hospitalized for RSV in this study had such low preexisting antibody titers allowed us to analyze antibody responses in young infants without large amounts of passively acquired maternal antibody. Thus, it may be that the mechanisms postulated to mediate antibody-induced suppression of the humoral response—such as epitope masking, inhibition of B cell activation through co-cross-linking of FcγRIIB and the B cell receptor, and Fc receptor–mediated phagocytosis of antigen-antibody complexes [44]—may be more influential than age in determining whether RSV infection induces an antibody response in young infants.

Our study has several limitations. We evaluated antibody responses to RSV infection in Navajo and WMA infants and children, and the strong primary responses we observed may be specific to these populations. Although this seems unlikely, it will be important to corroborate our findings regarding neutralizing antibody responses in other populations of young infants with low levels of passively acquired antibody. It is reassuring to

note that Navajo and WMA infants and children were similar to other populations in terms of the differences noted between neutralizing antibody titers in hospitalized infants and those in the community, suggesting that, as in other populations, neutralizing antibody has a protective effect in these American Indian infants. In addition, our study involved natural infection with wt RSV and evaluation of hospitalized infants whose infections were detected by rapid antigen assay, which might have selected for infants with particularly high RSV loads. In general, hospitalized infants shed high titers of RSV that should be detectable by rapid antigen assay [45]. However, it will be important to determine whether the viral loads associated with hospitalization due to RSV infection are required to induce high titers of neutralizing antibody in infants with low titers of preexisting antibody or whether these types of responses can occur after milder outpatient RSV infections with lower viral loads or after infection with live attenuated RSV vaccine candidates that are even more restricted in replication.

What are the implications of this study for the future development of RSV vaccines? First, infants and children developed strong antibody responses to both F and G proteins, indicating that both antigens should be represented in a live attenuated vaccine. Second, infants and children also developed strong and consistent antibody responses to the highly conserved cysteine noose region of RSV G, suggesting that a bivalent live attenuated vaccine containing 1 RSV A and 1 RSV B strain could protect against strains with changes in the variable regions of RSV G. Third, although preclinical studies of live attenuated recombinant RSV vaccines that lack the cysteine noose have been recently described [46], the present study, along with previous preclinical [38, 41] and clinical [42] studies, highlights the importance of the cysteine noose region of RSV G as part of a live attenuated RSV vaccine. Finally, it appears that very young infants with low titers of maternally derived antibody can mount a substantial RSV neutralizing antibody response under conditions in which the immunosuppressive effect of maternal antibodies is reduced. This suggests that immunosuppression by maternally derived antibodies rather than immunologic immaturity is the major obstacle to the development of a strong humoral response against RSV infection during infancy.

A live attenuated RSV vaccine would likely be given at multiple intervals during the first 6 months of life [2, 47], which might ensure the development of protective antibody responses at critical time points for individual infants. With the first dose of vaccine administered at 1–2 months, responses may be observed only in infants with low titers of antibody, but high titers of passively acquired antibody would likely protect the nonresponders against severe RSV disease. With subsequent doses of vaccine, increasing proportions of infants might respond as maternally derived antibody wanes. Phase 2 and 3 studies of live attenuated RSV vaccine candidates in young infants are needed to test this hypothesis.

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